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**Analysis of DNA methylation in young people reveals
limited evidence for an association between victimization stress
and epigenetic variation in blood**

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ABSTRACT (253 words)

Objective: To test if victimization stress is associated with genome-wide DNA methylation. DNA methylation has been proposed as an epigenetic mechanism by which early-life experiences become “embedded” in the genome and alter transcriptional processes to compromise health.

Method: We tested the hypothesis that victimization is associated with DNA methylation in the Environmental Risk (E-Risk) Longitudinal Study, a nationally representative 1994-1995 birth cohort of 2232 twins born in England and Wales and assessed at ages 5,7,10,12, and 18 years. Multiple forms of victimization were ascertained in childhood and adolescence (including physical, sexual and emotional abuse, neglect, exposure to intimate-partner violence, bullying, cyber- and crime victimization).

Results: Epigenome-wide analyses of polyvictimization across childhood and adolescence revealed few significant associations with DNA methylation in peripheral blood at age 18 years, but these were confounded by tobacco smoking and/or did not survive co-twin control tests. Secondary analyses of specific forms of victimization revealed sparse associations with DNA methylation that did not replicate across different operationalizations of the same putative victimization experience. Hypothesis-driven analyses of six candidate genes in the stress response (*NR3C1*, *FKBP5*, *BDNF*, *AVP*, *CRHR1*, *SLC6A4*) did not reveal predicted associations with DNA methylation in probes annotated to these genes.

Conclusion: Findings from this epidemiological analysis of the epigenetic effects of early-life stress do not support the hypothesis of robust changes in DNA methylation in victimized young people. We need to come to terms with the possibility that epigenetic epidemiology is not yet well matched to experimental, non-human models in uncovering the biological embedding of stress.

INTRODUCTION

Early-life stress is thought to cause many adverse health outcomes, including altered brain development(1), compromised cognitive functioning(2) poor mental health(3), and multiple physical illnesses(4). Perhaps the most pressing question at the nexus of neurobiology and public health is how stress “gets under the skin” to bring about these pleiotropic effects(5-9). Although the molecular mechanisms linking early-life exposures to these multiple outcomes are not yet understood, vigorous scientific attention is currently focussed on the role of long-term alterations to gene expression and function, mediated by dynamic epigenetic modifications(10). In particular, DNA methylation has been proposed as a mechanism by which early-life experiences may become “embedded” in the genome (11) and this possibility has captured the public imagination as a means by which stress becomes toxic, damaging learning, behavior, and health across the lifespan (Center on the Developing Child; <http://developingchild.harvard.edu/science/key-concepts/toxic-stress/>).

Research into the epigenetic consequences of early-life adversity was spurred by experimental studies documenting that variation in maternal care in early life was associated with epigenetic alterations in the brains of rodents (12). Translation to humans has not been unchallenging. Although a substantial body of research has accumulated linking early-life adversity to differences in DNA methylation, methods and results are heterogeneous and non-overlapping. Under the label of ‘adversity’, studies have focused on a diverse mix of exposures spanning maternal psychiatric disease (13), early parental loss(14), institutionalization(15), indentured child labor(16), child abuse(17), and the Holocaust(18). On the outcome side, DNA methylation differences in

surrogate tissue, such as whole blood, have been reported, but often in different genomic regions. Moreover, because it is ethically impermissible to randomly assign human participants to varying levels of adversity, observational studies have struggled to disentangle the effects of stress exposure from confounding effects of other toxins (e.g., tobacco smoking) and a host of other environmental (e.g., poverty) and genetic factors known to be correlated with stress exposure. More investigation of the link between stress exposure and DNA methylation is needed.

In this article, we report a test of the hypothesis that victimization is associated with DNA methylation in whole blood in a population-representative longitudinal study of a twin birth cohort, followed to age 18 years. The study was explicitly designed--rather than conceived post hoc--to test for genomic consequences of victimization. To ensure the content validity of the measurement of threatening and violating stressful experiences, the study canvassed the multiple types of victimization that the young study participants encountered during development, both inside and outside the family, including physical and sexual abuse, emotional and physical neglect, and, as they grew older, bullying, cyber-victimization, and crime. Victimization was measured during adolescence (to test the impact of adverse events during the peak-prevalence period of victimization), during childhood (to test the sensitive period of early-life adversity), and cumulatively across the first two decades of life (to test the cumulative stress-load hypothesis of repeated and chronic stress). Using these exposure data, we report a two-pronged approach to discovery research on the epigenetics of stress. First, we conducted an epigenome-wide association study (EWAS) to test the hypothesis that victimization experiences are linked to methylation variation. Second, we interrogated

variation in DNA methylation in the vicinity of candidate genes previously implicated in the stress response.

METHODS

Sample

Participants were members of the Environmental Risk (E-Risk) Longitudinal Twin Study, which tracks the development of a 1994-95 birth cohort of 2,232 British children(19). Briefly, the E-Risk sample was constructed in 1999-2000, when 1,116 families (93% of those eligible) with same-sex 5-year-old twins participated in home-visit assessments. This sample comprised 56% monozygotic (MZ) and 44% dizygotic (DZ) twin pairs; sex was evenly distributed within zygosity (49% male). The study sample represents the full range of socioeconomic conditions in Great Britain, as reflected in the families' distribution on a neighborhood-level socioeconomic index (ACORN [A Classification of Residential Neighbourhoods], developed by CACI Inc. for commercial use) (20): 25.6% of E-Risk families live in “wealthy achiever” neighborhoods compared to 25.3% nationwide; 5.3% vs. 11.6% live in “urban prosperity” neighborhoods; 29.6% vs. 26.9% in “comfortably off” neighborhoods; 13.4% vs. 13.9% in “moderate means” neighborhoods; and 26.1% vs. 20.7% in “hard-pressed” neighborhoods. E-Risk underrepresents “urban prosperity” neighborhoods because such households are often childless.

Home visits were conducted when participants were aged 5, 7, 10, 12 and most recently, 18 years (93% participation). Our epigenetic study used DNA from a single tissue: whole blood. At age 18, whole blood was collected in 10mL K₂EDTA tubes from 1,700 participants and DNA extracted from the buffy coat. (Study members who did not

provide blood provided buccal swabs, but these were not included in our methylation analysis to avoid tissue-source confounds.) There were no differences between participants who did versus did not participate and who did versus did not provide blood in terms of their socioeconomic background, IQ, mental health, or victimization experiences. Details are in the **Supplementary Information**.

Genome-wide quantification of DNA methylation

We assayed 1669 blood samples (out of 1700); 31 samples were not useable (e.g., due to low DNA concentration). ~500ng of DNA from each sample was treated with sodium bisulfite using the EZ-96 DNA Methylation kit (Zymo Research, CA, USA). DNA methylation was quantified using the Illumina Infinium HumanMethylation450 BeadChip (“Illumina 450K array”) run on an Illumina iScan System (Illumina, CA, USA). Twin pairs were randomly assigned to bisulfite-conversion plates and Illumina 450K arrays, with siblings processed in adjacent positions to minimize batch effects.

Data were imported using the *methylumIDAT* function in *methylumi*(21) and subjected to quality control analyses, checking for sex mismatches, genotype data that did not concur with those typed on Illumina OmniExpress24v1.2 arrays, and excluding low intensity samples (details in **Supplementary Information**).

Samples from 1658 participants passed our QC pipeline, including 1468 participants who were members of complete twin pairs (430 MZ pairs and 304 DZ pairs) and 190 participants whose co-twin did not have complete data (e.g., did not provide blood, did not pass QC). Data were processed with the *pfilter* function from the *wateRmelon* package(22) excluding 0 samples with >1% of sites with a detection p value >0.05, 567 sites with beadcount <3 in 5% of samples and 1448 probes with >1%

of samples with detection p value >0.05. The data were normalized with the *dasen* function from the *watermelon* package(22). Prior to any analyses, probes with common (>5% MAF) SNPs within 10 bp of the single base extension and probes with sequences previously identified as potentially hybridizing to multiple genomic loci were excluded (23, 24), resulting in a final dataset of 430,802 probes.

Victimization Exposure

Childhood and adolescent victimization experiences in this cohort have been described previously(2, 25) and are briefly summarized here (details in **Supplementary Information**).

Childhood victimization was assessed repeatedly when the children were 5, 7, 10, and 12 years of age, including: exposure to intimate-partner violence between the mother and her partner; frequent bullying by peers; physical maltreatment by an adult; sexual abuse; emotional abuse and neglect; and physical neglect. Exposures were coded from 12-year dossiers for each child that comprised information from home-visit staff, mothers, children, family doctors, and child-protection interventions (**Supplementary Information**). Each exposure across childhood was coded on a 3-point scale: 0=no exposure; 1=probable/less severe exposure; 2=definite/severe exposure. Polyvictimization refers to the experience of multiple victimizations of different types, and it is a more powerful predictor of adverse outcomes than any particular exposure(26). Following Finkelhor et al.(26), this study used the most straightforward and reproducible method to define poly-victimization, operationalized as the simple count of different forms of victimization experienced by a child. All childhood victimization experiences coded as 2 were summed: 1,192 (71.9%) children had zero

severe victimization experiences; 355 (21.4%) had 1; 70 (4.2%) had 2; 41 (2.5%) had 3 or more severe victimization experiences.

Adolescent victimization was assessed at age 18 years when the twins were interviewed about experiences between 12-18 years using the Juvenile Victimization Questionnaire (JVQ)(27, 28), adapted as a clinical interview. Age 12 is a salient age for our participants because it is when British children leave primary school to enter secondary school. The JVQ has good psychometric properties(29) and was used in the U.K. National Society for the Prevention of Cruelty to Children national survey(30, 31), thereby providing benchmark values for comparisons with our cohort. The study assessed 7 different forms of victimization: maltreatment, neglect, sexual victimization, family violence, peer/sibling victimization, cyber victimization, and crime victimization. Like childhood victimization, exposure to each type of adolescent victimization was coded on a 3-point scale: 0=no exposure; 1=less severe exposure; 2=severe exposure (**Supplementary Information**). Adolescent polyvictimization was derived by summing all victimization experiences coded as “2”: 1,064 (64.2%) adolescents had zero severe victimization experiences; 325 (19.6%) had 1; 150 (9.2%) had 2; 118 (7.1%) had 3 or more severe victimization experiences.

Cumulative victimization. We performed a Latent Class Analysis combining the above-mentioned childhood and adolescent measures of victimization. Latent Class Analysis is a person-centered technique that classifies individuals into groups based on a profile of variables, in this case the degree of each participant’s exposure (i.e., none, moderate, or severe) to the six types of childhood and seven types of adolescent victimization. The Latent Class Analysis was performed using only participants who

experienced at least one form of victimization, and conducted in MPlus v7.4 accounting for clustering of twins within families (**Supplementary Table 1**). The Latent Class Analysis identified three victimized groups: Individuals who were (a) exposed primarily to parental intimate-partner violence in childhood (n=254; 15%); (b) primarily victimized by peers and 'street crime' throughout childhood and adolescence (n=412; 24.8%); or (c) experienced multiple types of violence in both childhood and adolescence (n=158; 9.5%). For the analysis of cumulative victimization, we report a comparison of the most extreme groups from the 1658 participants with methylation data: the 158 young people who were exposed to cumulative victimization across both childhood and adolescence versus the 834 (50.3%) young people who were not exposed to childhood or adolescent victimization.

Twins' retrospective self-reports of maltreatment during childhood. In addition to the above prospectively-ascertained measures of victimization, we assessed twins' recall of victimization up to age 12 years through the Childhood Trauma Questionnaire (CTQ)(32), completed by participants at the age-18 follow-up. The CTQ inquires about the severity of 5 forms of victimization: physical abuse, physical neglect, emotional neglect, sexual abuse, and emotional abuse. 1,507 (91%) participants recalled zero moderate/severe victimization experiences; 97 (5.9%) 1; 25 (1.5%); and 25 (1.5%) recalled 3 or more severe victimization experiences.

Statistical Methods

Linear regression was used to test the association between victimization and DNA methylation variation. The model included the following covariates: sex, methylation-array control-probe principal components indexing technical variation, and

cell-type proportion estimates (**Supplementary Information**). To control for known effects of smoking in methylation data, the model was re-fitted by adding information about smoking status as a covariate. Because the sample included members of twin pairs, we accounted for the non-independence of observations by calculating robust standard errors using the R package 'gee'. Complete results files showing associations between victimization measures and each probe are available for readers to consult in an **Online Data Supplement**. [For now this is hosted at Duke Box (<https://duke.box.com/s/2x6vp2gsp2emnwd6jcvcshtw2pvsw88>), password = CaspiVictimizationMethylation.]

We used regression to test the association between within-pair twin differences in victimization and within-pair differences in their DNA methylation, controlling for differences in blood cell-type proportion estimates and differences in smoking.

An array-wide significance threshold of $P < 1.16 \times 10^{-7}$ was derived by applying a Bonferroni-correction to the nominal $\alpha = 0.05$ threshold, thereby adjusting for the 430,802 probes tested in the study.

We also interrogated candidate genes hypothesized to be involved in stress reactivity by identifying probes on the array that were annotated to prespecified genes. Probe sequences for the Infinium HumanMethylation450 BeadChip kit were aligned to the hg19 version of the human genome using the BLAT (33) alignment algorithm. Probe sequences that mapped to multiple genomic loci were assigned to the genomic location provided by Illumina. Probe sequences that did not match any region of the genome with at least 94% identity were also assigned to the genomic location provided by Illumina. Each probe was then assigned to its nearest gene based on the

GRCh37v75 ENSEMBL(34) release of the human transcriptome. We report associations between victimization and these probes using both array-wide and gene-wide P -value thresholds.

Regional Manhattan plots were generated using R packages ‘*qqman*’ and ‘*ggplot2*’ and Bioconductor package ‘*ggbio*’.

RESULTS

Victimization in the peak period of adolescence

Adolescents are victimized by a more diverse set of actors across a wider range of environments than any other age group, and exposure to multiple types of victimization—including relational aggression, sexual victimization, and serious violent crime—peaks during adolescence(35). Therefore, we first tested whether polyvictimization during this peak period of exposure was associated with DNA methylation. Adolescent polyvictimization was significantly associated with DNA methylation ($P < 1.16 \times 10^{-7}$) at three differentially methylated positions (cg05575921, cg26703534 and cg21161138 all annotated to *AHRR*) (**Figure 1A**). Across all probes on the array, effect sizes were small (**Online Data Supplement**) and these three probes were characterized by DNA methylation differences $< 1\%$ for each additional type of victimization.

A prominent challenge to interpreting associations between victimization and DNA methylation is that victimized adolescents are more likely to smoke tobacco(36) which has striking effects on DNA methylation(37). We assessed smoking among E-Risk participants by calculating the number of “pack-years” that they smoked, observing that victimized adolescents were significantly more likely to smoke ($P = 2.51 \times 10^{-37}$), and

to have smoked more pack-years ($P=4.92\times 10^{-14}$, **Figure 2A**). In E-Risk we also replicate the finding that tobacco smoking is associated with multiple genome-wide changes in DNA methylation, with 83 probes meeting the array-wide significance threshold (**Figure 2B**; see **Supplementary Table 2** for more details). When smoking pack-years was added as a covariate to the polyvictimization EWAS, no probes remained significantly associated with polyvictimization at an array-wide significance threshold (**Figure 1B**). This is not surprising, because the three array-wide significant probes from the polyvictimization EWAS--those annotated to *AHRR*—were also among the probes significantly associated with tobacco smoking in a large recent EWAS(37) as well as in the EWAS of pack-years in E-Risk (**Figure 2C**).

Of course, not all types of victimization are alike. Some involve physical injury whereas others involve psychological insult; some are immediate, others remote (e.g., cyberbullying); some are perpetrated by strangers, others by people known to the victim. Our measure of polyvictimization may have diluted the effects of specific forms of victimization. Thus, we next tested the association between exposure to each victimization type (i.e. maltreatment, neglect, sexual victimization, family violence, peer/sibling victimization, cyber victimization, and crime victimization) and DNA methylation (**Supplementary Figure S1**). The results revealed few novel associations and sparsely distributed significant findings across the seven types of victimization. We detected a total of eight array-wide significant associations, but none of the eight were shared between victimization types. Two of the eight (cg05575921 and cg21161138, both annotated to *AHRR* and confounded by smoking) were among the three previously identified in our analysis of adolescent polyvictimization.

Testing the sensitive period of childhood victimization

Although most victimization experiences peak in adolescence, it has been hypothesized that the most biologically consequential victimization is experienced earlier in life(38). However, no probes passed the array-wide significance threshold in a regression model of childhood polyvictimization (**Figure 3**).

As with adolescent polyvictimization, we tested associations between exposure to each of the six victimization types (i.e., physical abuse, physical neglect, emotional abuse/neglect, sexual abuse, intimate-partner violence, and bullying victimization) and DNA methylation (**Supplementary Figure S2**). A total of 48 array-wide significant associations were observed across four of the six victimization types (physical abuse, emotional abuse/neglect, sexual abuse, and intimate-partner violence). None of these probes were shared between victimization types nor were they identified in the EWAS of childhood polyvictimization. Interestingly, of these 48 probes, 39 were associated with childhood sexual victimization. These probes are listed in **Supplementary Table 3**. These findings indicate that childhood sexual victimization is associated with stable DNA methylation differences in whole blood in young adulthood. However, these findings should be interpreted with caution because few children had recorded sexual victimization (n=29) and these associations were not observed in relation to sexual victimization in adolescence (**Supplementary Table 3**).

Testing the cumulative stress load hypothesis

Perhaps the most consequential stressors are those that are experienced chronically or recurrently. In particular, revictimization is a striking finding in epidemiology(39). For example, in E-Risk, every type of victimization in childhood was

associated with a significantly greater risk of victimization in adolescence(25), and polyvictimized children were 1.60 (95% CI:1.42-1.82) times more likely to be polyvictimized again as adolescents (RR=1.60, 95% CI:1.42,1.82). This suggested the hypothesis that the biological embedding of victimization is especially likely to occur in response to a greater cumulative stress load. The latent class representing cumulative polyvictimization was significantly associated with four CpG probes (cg05575921 and cg21161138 both annotated to *AHRR*, cg00944304 annotated to *BAHD1*, and cg03636183 annotated to *F2RL3* (**Figure 4A**). Two of these were the same *AHRR* probes previously found to be associated with adolescent polyvictimization (see Figure 1, Panel A). None of these probes remained array-wide significant after controlling for smoking (**Figure 4B**).

Are retrospective reports of childhood victimization in young adulthood associated with epigenetic variation?

Some evidence linking childhood adversity with DNA methylation comes from cross-sectional studies of adults who retrospectively report on their childhood experiences(40). Moreover, some research suggests that the association between childhood maltreatment and mental-health problems is stronger when maltreatment is retrospectively recalled than when it is ascertained prospectively(41, 42). We thus extended our analysis to test whether associations would emerge when childhood victimization was measured at age 18 with the Childhood Trauma Questionnaire(32), a popular tool for retrospectively assessing childhood maltreatment history in adults. Similar to what has been observed in other studies (43, 44), in E-Risk there was only fair agreement between prospective and retrospective reports of childhood

maltreatment (weighted kappa=.21), raising the possibility that different exposure operationalization may yield different findings.

Retrospective reports of childhood victimization were significantly associated with two probes, neither of which had been identified in any of the previous analyses (cg03960390 annotated to *RER1*; cg07146173 annotated to *ALKBH5*) (**Figure 5A**). After controlling for smoking, both remained array-wide significant (**Figure 5B**). We carried these two probes forward to a co-twin control analysis, which tested whether the more victimized twin was differentially methylated relative to their less-victimized co-twin. Neither of these probes remained statistically significant at $\alpha=.025$ (correction for two tests) in a co-twin control model (P s=.06 and .09, respectively), suggesting that the association between victimization and methylation is possibly confounded by shared genetic and/or environmental factors.

As with the other measures of polyvictimization, we tested associations between each of the five CTQ victimization types (i.e., physical abuse, physical neglect, emotional neglect, sexual abuse, and emotional abuse) and DNA methylation (**Supplementary Figure S3**). A total of 48 array-wide significant associations were observed across all five CTQ victimization types. None of these probes were shared between victimization types. Of these 48 probes, 22 were associated with retrospective reports of childhood sexual abuse. However, the probes associated with CTQ reports of childhood sexual abuse did not overlap with probes associated with prospectively-ascertained reports of childhood sexual victimization. **Supplementary Table 3** lists all the probes associated with sexual victimization (as measured during childhood, in adolescence, and retrospectively in young adulthood) and documents that DNA

methylation patterns associated with sexual victimization were not reproducible across different operationalizations of this stressor.

To further investigate associations between retrospective CTQ reports of childhood victimization and DNA methylation, we turned to comparable data from the Dunedin Longitudinal Study(45), which followed a 1972-73 birth cohort to age 38 years. At age 38 years, we administered the same CTQ measure as in E-Risk, assayed DNA methylation from blood samples using the same Illumina 450K array, and conducted parallel analyses of 818 Dunedin Study members with complete data (details in **Supplementary Information**). Two findings stand out. First, after controlling for smoking no probes passed the array-wide significance threshold in a regression of DNA methylation on retrospective CTQ reports of childhood victimization (**Supplementary Figure S4**). Second, none of the 22 probes that were associated with retrospective CTQ reports of childhood sexual abuse in E-Risk met the significance threshold in Dunedin (based on a Bonferroni correction for number of probes tested $[\text{.05}/22=\text{.002}]$) (**Supplementary Table 3**).

Epigenetic interrogation of known stress-related genes

An epigenome-wide analysis overlooks cumulative evidence about biological plausibility of specific candidate genes. In particular, genes involved in HPA-axis reactivity may be differentially methylated in response to victimization. These loci include *NR3C1* (the glucocorticoid receptor, which binds cortisol and triggers its downstream effects on gene expression)(46); *FKBP5* (a regulator of the glucocorticoid receptor network)(47); *BDNF* (the brain derived neurotrophic factor, a member of the nerve growth factor family)(48); *AVP* (the gene encoding the neuropeptide vasopressin,

which is secreted as part of the HPA response to stress)(49), and *CRHR1* (the corticotrophin releasing hormone receptor, another major player in the HPA pathway)(50). In addition, genetic variants annotated to *SLC6A4* (the serotonin transporter gene) have been implicated in stress reactivity and these effects may be mediated by altered DNA methylation(51).

We first identified all probes on the Illumina 450K array annotated to each of these six genes. The number of probes per gene ranged from 16 to 66. We then examined associations between these probes and adolescent, childhood, and cumulative victimization, as well as retrospective CTQ reports of childhood victimization.

To illustrate, **Figure 6** shows the regional Manhattan plot of $-\log_{10} P$ values and effect directions (negative [hypo-] vs. positive [hyper-] methylation) for all probes annotated to *NR3C1*. (The plot shows probes annotated to the genomic region surrounding *NR3C1* and the gene-wide significance threshold is based on a Bonferroni correction for the number of probes annotated to the gene.) For *NR3C1*, we observed no gene-wide significant probes associated with victimization within the *NR3C1* gene region. The regional Manhattan plots for the remaining five candidate genes are shown in **Supplementary Figures S5-S9**. Overall, looking across the six candidate genes and the four victimization exposures, only 4 probes crossed the threshold for gene-wide significance (*AVP* cg23035419 ($P=.0016$) and cg25551168 ($P=.0019$) in relation to adolescent polyvictimization; *BDNF* cg20954537 ($P=.0002$) in relation to CTQ reports of polyvictimization; *FKBP5* cg00140191 ($P=.0004$) in relation to childhood polyvictimization). Individual association statistics for all probes annotated to the six genes, in relation to each of the four victimization exposures, are listed in the **Online**

Data Supplement and the Figures show significance values for probes in the regions surrounding each gene, as well.

DISCUSSION

This study offers, to our knowledge, the most comprehensive analysis to date of epigenetic alterations in humans' response to victimization stress in the first two decades of life. The study design contains five strengths intended to enhance internal and external validity. First, it is the largest sample yet to test the association of victimization with epigenetic variation in whole blood. Second, it minimized both ascertainment and attrition bias; the study followed a nationwide birth cohort that represents the full range of socioeconomic conditions in Great Britain with no referral bias or selective attrition. Third, the study collected valid detailed measures of multiple forms of victimization in childhood and in adolescence, and ascertained cumulative victimization across the first two decades of life. Fourth, it introduced controls for smoking, which may generate spurious epigenetic associations with psychosocial factors. Fifth, it was able to use a family-based design to compare twin-siblings growing up in the same households to test confounding effects of shared environmental and genetic risk factors.

Results from both epigenome-wide association analyses and from interrogation of candidate genes involved in the human stress response revealed limited evidence for an association between victimization and epigenetic variation in peripheral blood. Three contributions to the literature stand out. First, relative to non-psychosocial toxins, such as tobacco (see Supplementary Table 2), methylation associations with “toxic” psychological stress are less pervasive and small. Second, methylation associations

identified for victimization overlapped with those identified for tobacco smoking, highlighting difficulties in disentangling biological effects of psychosocial influences from effects of health behaviors associated with a victimization history. Third, the few methylation associations that were identified for victimization did not replicate across different specifications of victimization stress, including ascertainment periods and reporting sources, nor in an additional replication sample.

Guided by the hypothesis that the epigenome mediates the effects of stress on poor health(52), epigenetic variation detected in blood is hypothesized to represent an informative window onto studying biological embedding(48). For this to be true, it would be important to observe epigenetic differences between victimized children and their non-victimized peers that were either moderate in size or pervasive across the genome. Results from this study are not consistent with the presence of such epigenetic differences in peripheral blood.

One of the most important questions regarding the present study is how the findings should be interpreted in the context of extant research about biological embedding of early-life stress and childhood maltreatment. This is particularly salient given reports that do document epigenetic influences of stressful experiences.

First, it is possible that the lack of associations observed in the present study constitute a false negative. However, we had invested heavily in this project, were surprised by the dearth of findings, and were motivated to turn our data upside down in an effort to detect significant associations. In addition to 'epigenome-wide' analyses, we carried out candidate-gene association analyses; we tested associations with victimization in different age periods as well as cumulative victimization across multiple

age periods; we disaggregated our index of polyvictimization in order to test associations with multiple different specific forms of victimization, including physical abuse, sexual abuse, and crime victimization; we used victimization data from self and others; we presented findings at different significance thresholds, ranging from array-wide p-values to gene-wide p-values. Against the background of these efforts, the results did not yield consistent reproducible findings.

Second, it is possible that our exposure measurement was inadequate, either due to unreliability or poor content validity. However, we used reliable, valid, and age-appropriate methods to assesses victimization, and exposed cases in our cohort were known to endure severe experiences. Moreover, the victimization experiences we analyzed predict poor psychiatric outcomes strongly in this cohort(3), attesting that it is possible to detect stress-related consequences of victimization using this study's victimization measures. Specifically, victimization was followed by increased mental-health problems over a childhood pre-victimization baseline of emotional/behavioral problems, and discordant-twin analyses showed that victimization predicted increased risk of mental-health problems independent of family background and genetic risk. On the outcome side, our methylation data replicate the same clear signatures of cigarette smoking that have been observed in other studies(37) (see Supplementary Table 2), attesting that it is possible to detect epigenetic modifications related to environmental exposures using this study's methylation data.

Third, it is possible that previous reports contain some false positive findings and/or more circumscribed findings. Many epigenetic studies of stress are very small, running the risk that effect sizes for statistically significant associations could be over-

estimated(53). Other studies do not always correct significance thresholds for multiple testing when evaluating epigenetic associations, or filter the probes in ways resulting in too-lenient correction. And, in some instances, positive epigenetic associations of early-life stress sometimes depend on genotype, on trauma exposure, or on the presence of psychiatric disorder(47, 54-56). In general, the replication record of previous findings is difficult to evaluate and summarize because different studies have examined different organisms, different age groups, different stressors, and different genomic regions sometimes in different tissues (brain, blood, saliva, or buccal cells). Additionally, variation in technological approaches introduces lack of parallelism in the type of methylation data produced (e.g., high-throughput array-based technologies vs. lower throughput pyrosequencing). Although there is an overall impression of links between childhood adversity and epigenetic variation, the consistency may be more apparent than real.

The lack of association in this large epidemiological study between victimization and epigenetic variation in blood raises several considerations for evaluating future studies about the epigenetics of stress.

First, seminal epigenetic research on biological embedding of stress used brain tissue in rodents(12), focusing on genomic regions of interest. However, with notable exceptions(46, 57), the vast majority of subsequent human studies, including the present one, have relied on peripheral blood. This choice is expedient, but also scientifically reasonable given the aim of detecting effects on stress-related physical health systems that include peripheral circulating processes (immune, neuroendocrine). But whole blood is heterogeneous, and although cell-type composition can be evaluated

and controlled, as in the present study, it does beg the question of whether peripheral blood is a problematic surrogate tissue for research on the epigenetics of stress(58). Comparisons of methylomic variation across blood and brain suggest that blood-based EWAS may yield limited information relating to underlying pathological processes for disorders where brain is the primary tissue of interest(59).

Second, the present study tested the ‘main-effect’ hypothesis that victimization experiences in childhood lead to epigenetic modifications. It is quite possible that the answer to this question is more nuanced. For example, exposure-related methylation signatures might be concentrated only among the subset of victimized individuals who develop stress-related disorders (60) or could be enhanced by risk factors or mitigated by protective factors(47). If the epigenetic effects of stress are so nuanced, the challenge will be in drawing conclusions from studies of a range of disorders and of multiple different risk and protective factors.

Third, researchers who seek to survey DNA methylation across the epigenome are limited to using commercial arrays whose probes only interrogate a proportion of all CpG sites in the epigenome. In this regard, it is important to note that key areas of the epigenome reported to index maltreatment exposure are sparsely covered on the Illumina array (40, 61). For example, no probes from the Illumina platform map to intron 7 of *FKBP5*(47), and the few probes which map to the *NR3C1* non-coding promoter region(46) are not significant in our study. Thus, our absence of evidence for methylation differences in candidate genes that have been associated with victimization does not translate to evidence of the absence of methylation differences in these or other genes. Moreover, while epigenetic modifications may play a mediating role in

translating and establishing the long-term health outcomes associated with early life stress and victimization, DNA methylation may not be the only epigenetic modification directly involved in this process(62, 63). More generally, it is important not to lose sight of the well-documented association between early-life victimization and poor adult health(8, 9), and of the need to uncover how this link comes about not only through various biological but also through psychological and life-style factors.

Fourth, observational studies are perennially challenged in getting a handle on the temporal nature of biological effects of stress. Unlike in experimental studies in model organisms—where stress can be manipulated (e.g., through glucocorticoid administration or maternal separation) and its epigenetic effects monitored at pre-set intervals—in epigenetic epidemiological studies of human stress, including the present one, stress exposure is often correlated with genetic differences, methylation variation is typically assessed at one point in time, and the gap between exposure and biological measurement is highly variable and often indeterminate(64). Environmentally-induced DNA modifications do not necessarily persist throughout the life course(65), and it is not known when and for whom epigenetic modification becomes biologically stabilized following stress exposure. This creates both design and interpretive challenges in documenting and describing dynamic responses to early childhood adversities.

In summary, findings from this comprehensive epidemiological analysis of the epigenetic effects of early-life stress did not support the hypothesis of robust changes in DNA methylation in victimized young people. Our conclusion is not that DNA methylation is unimportant. Rather, it is that observational studies of free-ranging humans relying on peripheral tissue and using currently available high-throughput

technologies appear to yield weak and inconsistent evidence about the epigenetics of early-life stress. We need to come to terms with the possibility that epigenetic epidemiology is not yet well matched to experimental, non-human models in uncovering how stress gets under the skin in humans.

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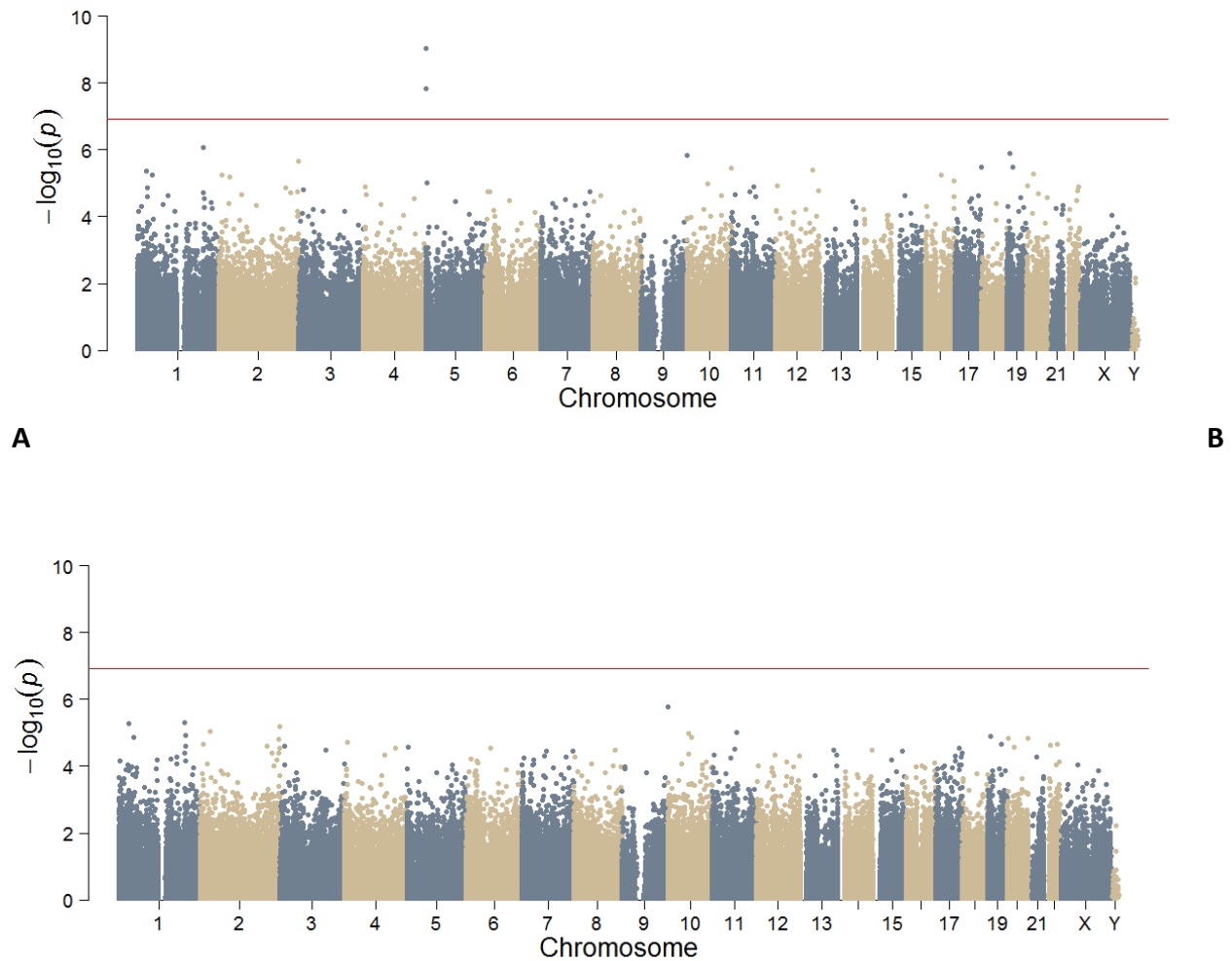
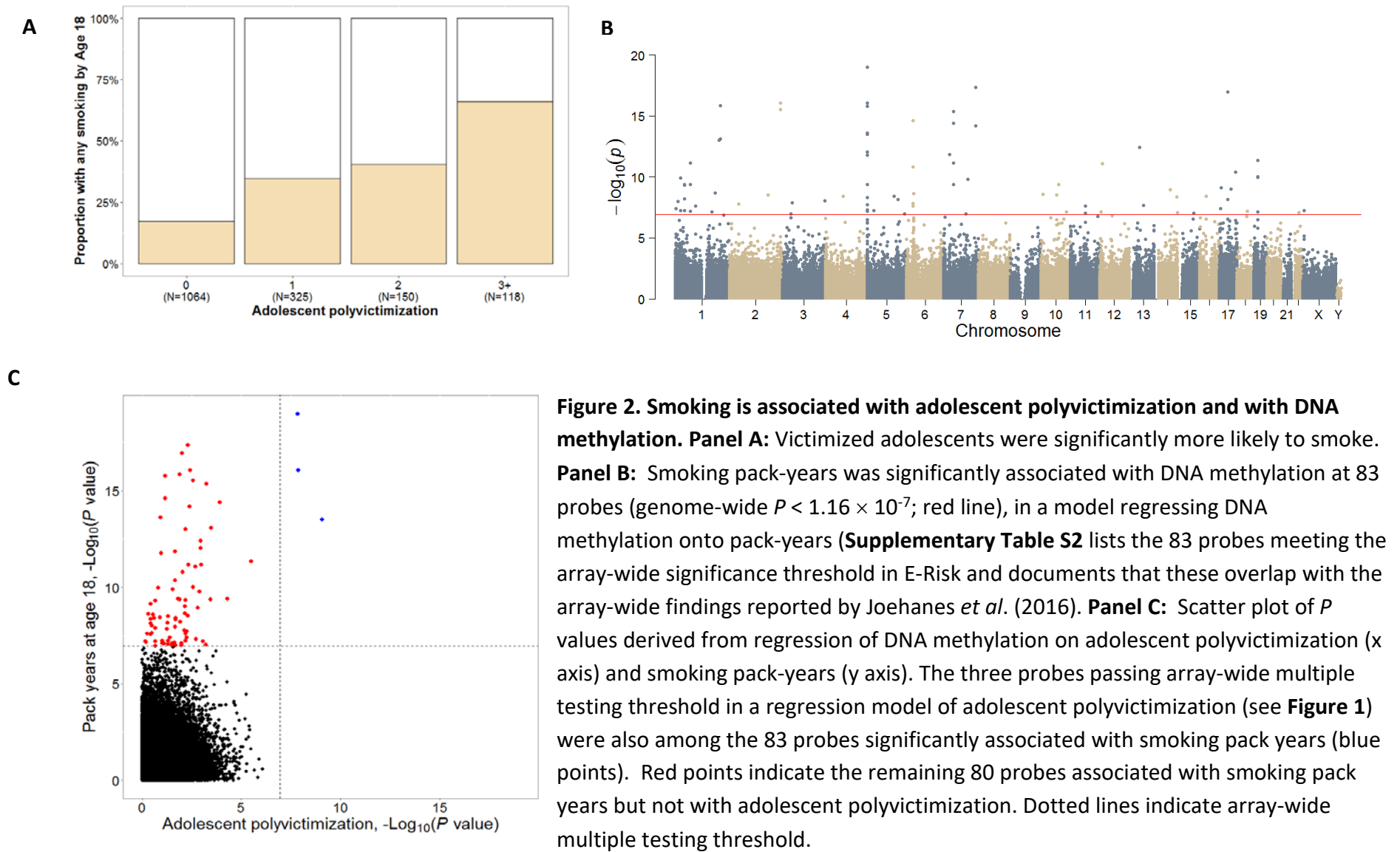


Figure 1. The association between adolescent polyvictimization and DNA methylation. Panel A: Three probes on chromosome 5 (annotated to *AHRR*) passed the array-wide multiple testing threshold ($P < 1.16 \times 10^{-7}$; red line). Note two of these probes are within 4Kb of one another. **Panel B:** We identified no significant associations when adding smoking pack-years as a further covariate, suggesting that the association between adolescent polyvictimization and DNA methylation is confounded by smoking.



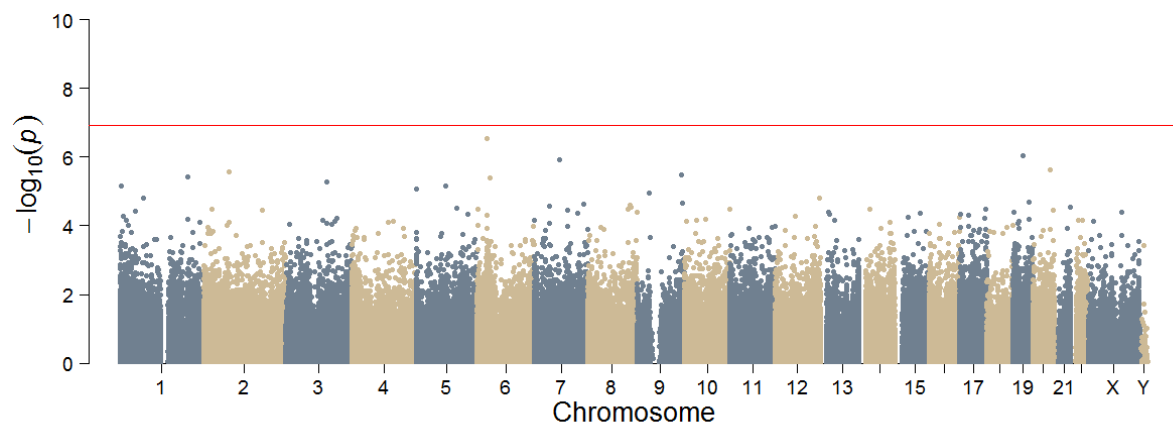
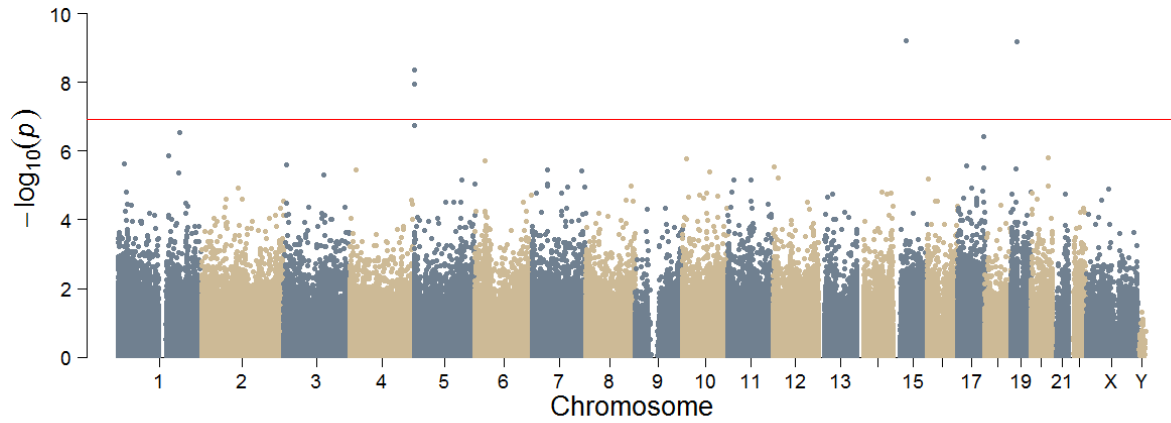


Figure 3. The association between childhood polyvictimization and DNA methylation. No probes passed array-wide multiple testing threshold.

A



B

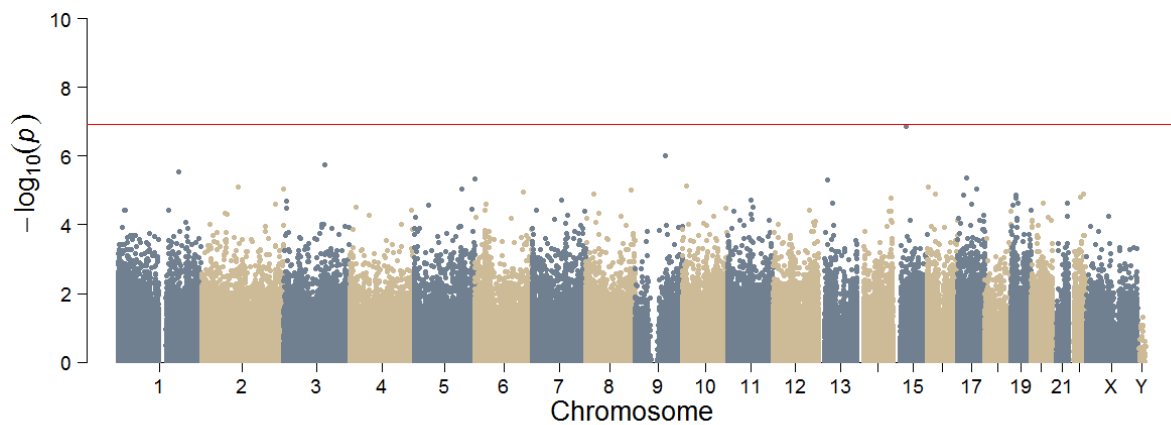


Figure 4. The association between cumulative victimization and DNA methylation. Panel A: Four probes passed the array-wide multiple testing threshold ($P < 1.16 \times 10^{-7}$; red line). **Panel B:** We identified no significant associations when adding smoking pack-years as a further covariate, suggesting that the association between cumulative victimization and DNA methylation is confounded by smoking.

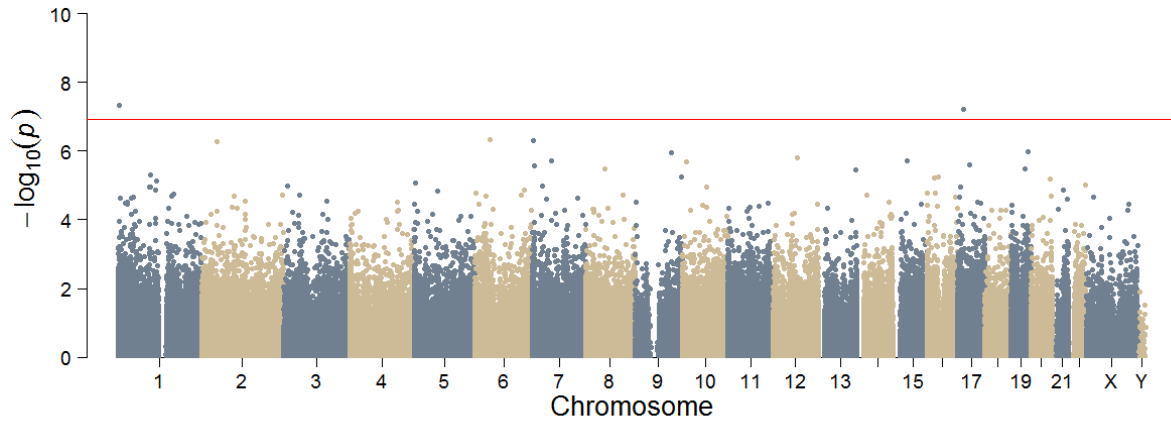
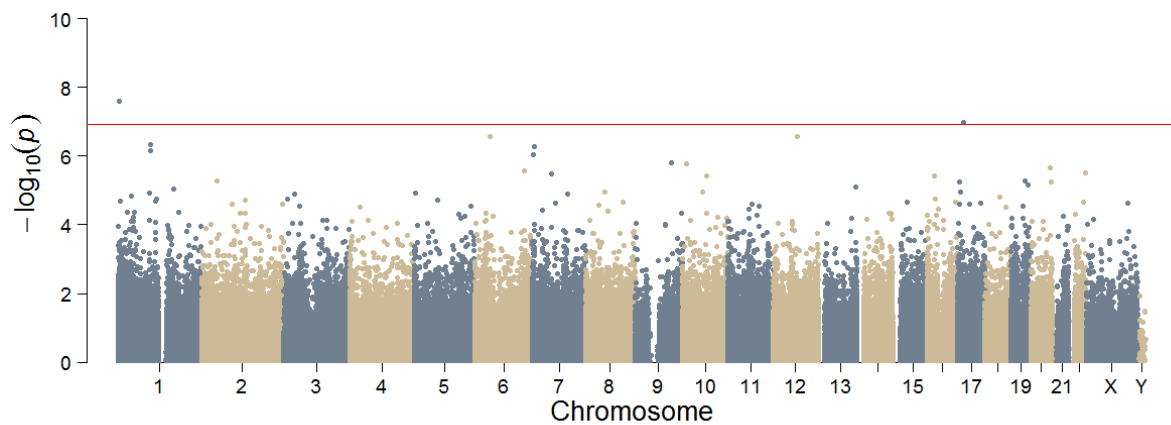
A**B**

Figure 5. The association between retrospective reports of childhood victimization on the Childhood Trauma Questionnaire and DNA methylation. Panel A: Two probes passed the array-wide multiple testing threshold ($P < 1.16 \times 10^{-7}$; red line), neither of which had been identified in any of the previous analyses. **Panel B:** The two probes remained array-wide significant after controlling for smoking.

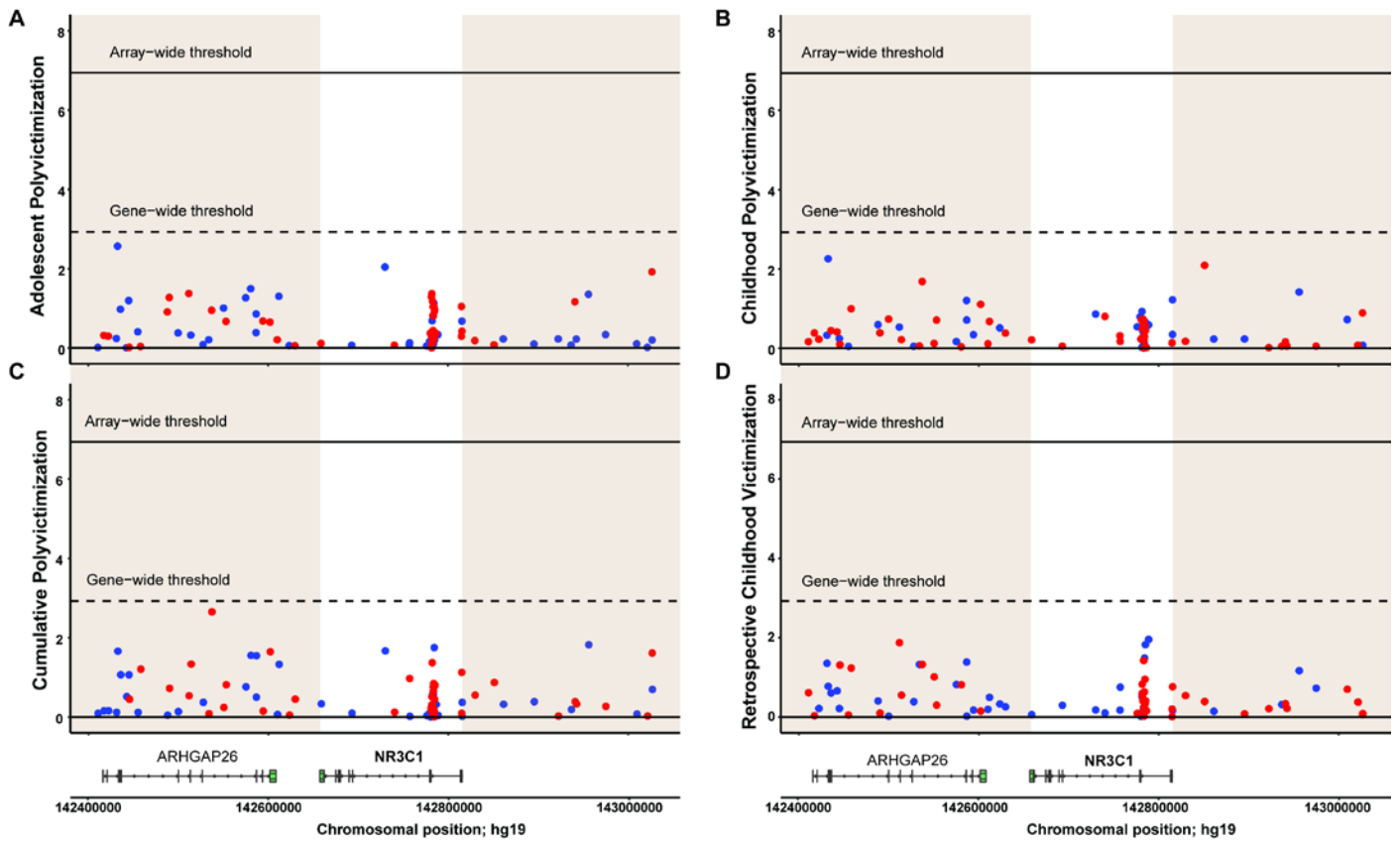


Figure 6. The association between victimization and DNA methylation across probes annotated to *NR3C1*. Panels **A-D** show regional Manhattan plots of $-\log_{10} P$ values for all probes annotated to the genomic region surrounding *NR3C1* in analyses of **(A)** adolescent polyvictimization, **(B)** childhood victimization, **(C)** cumulative victimization, and **(D)** retrospective reports of childhood victimization. The Panels show both the array-wide multiple testing threshold ($P < 1.16 \times 10^{-7}$) and the gene-wide significance threshold based on a Bonferroni correction for the number of probes annotated to the gene ($N=42$). Effect directions are indicated in blue (● hypomethylated) and red (● hypermethylated) circles. The white areas highlight probes localized to the genomic coordinates for *NR3C1*. The shaded areas highlight probes annotated to the *NR3C1* gene region (± 250 Kb up- and downstream of the gene), with diagrammatic gene annotations below for illustrative purposes.